

Synergy of Nitric Oxide and Silver Sulfadiazine against Gram-Negative, Gram-Positive, and Antibiotic-Resistant Pathogens

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Abstract: The synergistic activity between nitric oxide (NO) released from diazeniumdiolate-modified proline (PROLI/NO) and silver(I) sulfadiazine (AgSD) was evaluated against *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* using a modified broth microdilution technique and a checkerboard-type assay. The combination of NO and AgSD was defined as synergistic when the fractional bactericidal concentration (FBC) was calculated to be <0.5. Gram-negative species were generally more susceptible to the individual antimicrobial agents than the Gram-positive bacteria, while Gram-positive bacteria were more susceptible to combination therapy. The in vitro synergistic activity of AgSD and NO observed against a range of pathogens strongly supports future investigation of this therapeutic combination, particularly for its potential use in the treatment of burns and chronic wounds.

Keywords: Antimicrobial; combination therapy; nitric oxide; silver sulfadiazine; synergy; topical

Introduction

The antimicrobial properties of silver and more specifically ionic silver (Ag^+) have been recognized and utilized for centuries. Nano- to micromolar concentrations of Ag^+ exhibit broad-spectrum bactericidal, fungicidal, virucidal, and protozoicidal activity,¹ bonding covalently to electron-donating groups (e.g., the sulfhydryl of cysteine) or electrostatically to negatively charged molecules (e.g., DNA). Most bacterial sites targeted by Ag^+ are proteinaceous, where alterations in amino acid residues lead to structural damage and disruption of replicative and metabolic processes, ultimately resulting in cell death.^{1–6} Evidence suggests that interactions with DNA also play an important role in the antimicrobial efficacy of Ag^+ .^{4,5} Generally applied as an external treatment, Ag^+ is delivered via silver compounds such as silver(I)

sulfadiazine (AgSD).⁷ Most causative pathogens related to burns and chronic wound infections are susceptible to the

- (1) Russell, A. D.; Hugo, W. B. Antimicrobial activity and action of silver. *Prog. Med. Chem.* **1994**, *31*, 351–371.
- (2) Bragg, P. D.; Rainne, D. G. The effect of silver ions on the respiratory chain of *Escherichia coli*. *Can. J. Microbiol.* **1974**, *20*, 883–889.
- (3) Dibrov, P.; Dzioba, J.; Gosink, K. K.; Häse, C. C. Chemiosmotic mechanism of antimicrobial activity of Ag^+ in *Vibrio cholerae*. *Antimicrob. Agents Chemother.* **2002**, *46*, 2668–2670.
- (4) Modak, S. M.; Fox, J. C. L. Binding of silver sulfadiazine to the cellular components of *Pseudomonas aeruginosa*. *Biochem. Pharmacol.* **1973**, *22*, 2391–2404.
- (5) Feng, Q. L.; Wu, J.; Chen, G. Q.; Cui, F. Z.; Kim, T. N.; Kim, J. O. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater. Res.* **2000**, *52*, 662–668.
- (6) Holt, K. B.; Bard, A. J. Interaction of silver (I) ions with the respiratory chain of *Escherichia coli*: An electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag^+ . *Biochemistry* **2005**, *44*, 13214–13223.

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levels of AgSD attainable topically.^{8,9} Fortunately, exposure to clinical levels of Ag⁺ typically does not pose a threat to human health, despite the broad range of reactivity.^{1,7}

Like Ag⁺, nitric oxide (NO) is a broad-spectrum antimicrobial agent that has a number of cellular targets, and the endogenous expression of NO has been conserved throughout higher organisms as the immune system's first-line defense against infection.^{10–13} Although NO can modify proteins and other biological macromolecules directly,¹⁴ it is a highly reactive radical and frequently combines with locally abundant small molecules such as oxygen (O₂) and superoxide (O₂[−]), generating an arsenal of reactive byproducts that include dinitrogen trioxide (N₂O₃) and peroxynitrite (ONOO[−]).^{10,15} Collectively these reactive species evoke potent antibacterial effects by rendering nitrosative and oxidative stresses to bacteria.^{10,15–19} In vitro, NO administered via soluble, small-molecule diazeniumdiolate NO donors, from nanoparticle delivery vehicles, and from NO-releasing xerogel coatings, has been shown to kill a range of pathogens.^{20–25}

Although the appropriate use of antimicrobials to treat infection is a beneficial practice, artificial pressures resulting from overuse, patient noncompliance, and widespread application have promoted the unnatural selection of inherently

resistant microbes.²⁶ Even resistance to broad-spectrum agents has been observed.^{7,27} To complicate matters, methods of gene sharing employed by bacteria promote the localized collection of resistance determinants, often on transferable plasmids, leading to the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) species.^{28,29} In recent years, a dramatic rise in the incidence of “super bugs”, or bacteria that are resistant to those antibiotics generally reserved as a last resort treatment option (e.g., vancomycin), has been observed.^{30,31} Unfortunately, the discovery of new antimicrobials progresses slowly while resistance factors to all clinically employed antimicrobial agents emerge rapidly,²⁶ creating a critical need for alternative approaches to treating infection.

Combination therapy is one strategy for stemming the emergence of resistant species.^{32–34} The concerted use of two or more biocides with different mechanisms of action decreases the likelihood that an organism will possess all the traits necessary to ensure its selection and survival.

- (7) Silver, S.; Phung, L. T.; Silver, G. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 627–634.
- (8) Carr, H. S.; Wlodkowski, T. J.; Rosenkranz, H. S. Silver sulfadiazine: in vitro antibacterial activity. *Antimicrob. Agents Chemother.* **1973**, *4*, 585–587.
- (9) Wlodkowski, T. J.; Rosenkranz, H. S. Antifungal activity of silver sulphadiazine. *Lancet* **1973**, *ii*, 739–740.
- (10) Fang, F. C. Host/pathogen interactions: Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest.* **1997**, *99*, 2818–2825.
- (11) Bogdan, C. Nitric oxide and the immune response. *Nat. Immunol.* **2001**, *2*, 907–916.
- (12) Foley, E.; O'Farrell, P. H. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. *Genes Dev.* **2003**, *17*, 115–125.
- (13) MacMicking, J.; Xie, Q. W.; Nathan, C. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **1997**, *15*, 323–350.
- (14) Mannick, J. B. Immunoregulatory and antimicrobial effects of nitrogen oxides. *Proc. Am. Thorac. Soc.* **2006**, *3*, 161–165.
- (15) Wink, D. A.; Mitchell, J. B. Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biol. Med.* **1998**, *25* (4), 434–456.
- (16) Moller, M. N.; Li, Q.; Lancaster, J. R. J.; Denicola, A. Acceleration of nitric oxide autooxidation and nitrosation by membranes. *Life* **2007**, *59*, 243–248.
- (17) Zaki, M. H.; Akuta, T.; Akaike, T. Nitric oxide-induced nitrate stress involved in microbial pathogenesis. *J. Pharmacol. Sci.* **2005**, *98*, 117–129.
- (18) Wink, D. A.; Kasprzak, K. S.; Maragos, C. M.; Elespuru, R. K.; Misra, M.; Dunams, T. M.; Cebula, T. A.; Koch, W. H.; Andrews, A. W.; Allen, J. S.; Keefer, L. K. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **1991**, *254*, 1001–1003.
- (19) Schapiro, J. M.; Libby, S. J.; Fang, F. C. Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8496–8501.
- (20) Ghaffari, A.; Miller, C. C.; McMullin, B.; Ghahary, A. Potential application of gaseous nitric oxide as a topical antimicrobial agent. *Nitric Oxide* **2006**, *14*, 21–29.
- (21) Rauli, R.; McElhaney-Feser, G.; Hrabie, J. A.; Cihlar, R. L. Antimicrobial properties of nitric oxide using diazeniumdiolates as the nitric oxide donor. *Recent Res. Dev. Microbiol.* **2002**, *6*, 177–183.
- (22) Keefer, L. K. Progress toward clinical application of the nitric oxide-releasing diazeniumdiolates. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 585–607.
- (23) Hetrick, E. M.; Schoenfisch, M. H. Antibacterial nitric oxide-releasing xerogels: Cell viability and parallel plate flow cell adhesion studies. *Biomaterials* **2007**, *28*, 1948–1956.
- (24) Hetrick, E. M.; Shin, J. H.; Stasko, N. A.; Johnson, B. A.; Wespe, D. A.; Holmuhamedov, E.; Schoenfisch, M. H. Bactericidal efficacy of nitric oxide-releasing silica nanoparticles. *ACS Nano* **2007**, *2*, 235–246.
- (25) Nablo, B. J.; Rothrock, A. R.; Schoenfisch, M. H. Nitric oxide-releasing sol-gels as antibacterial coatings for orthopedic implants. *Biomaterials* **2005**, *26*, 917–924.
- (26) Levy, S. B. *The Antibiotic Paradox*, 2nd ed.; Perseus: Cambridge, 2002.
- (27) Kliebe, C.; Nies, B. A.; Meyer, J. F.; Tolxdorff-Neutzing, R. M.; Wiedemann, B. Evolution of plasmid-encoded resistance to broad-spectrum cephalosporins. *Antimicrob. Agents Chemother.* **1985**, *28*, 302–307.
- (28) Hall, R. M.; Collins, C. M. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol.* **1995**, *15*, 593–600.
- (29) Levy, S. B. Multidrug resistance: a sign of the times. *N. Engl. J. Med.* **1998**, *338*, 1376–1378.
- (30) Hiramatsu, K. Vancomycin resistance in *Staphylococci*. *Drug Resist. Updates* **1998**, *1*, 135–150.
- (31) Michel, M.; Gutmann, L. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci: therapeutic realities and possibilities. *Lancet* **1997**, *349*, 1901–1906.
- (32) Cottarel, G.; Wierzbowski, J. Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol.* **2007**, *25*, 547–555.
- (33) Eliopoulos, G. M.; Moellering, R. C., Jr. Antimicrobial Combinations. In *Antibiotics in Laboratory Medicine*, 4th ed.; Lorian, V., Eds.; Williams & Wilkins: Baltimore, 1996; pp 330–396.
- (34) Eliopoulos, G. M.; Eliopoulos, C. T. Antibiotic combinations: Should they be tested. *Clin. Microbiol. Rev.* **1988**, *1* (2), 139–156.

Agents possessing a broad spectrum of antimicrobial action may both lower the probability of developing resistance and manage the polymicrobial burden typically found in topical infections.^{33,34} As smaller quantities of each drug are generally required in the application of combination therapy, dose-related toxicity experienced to a particular biocide may also be reduced.^{33,34} In the best case scenario, the combination of antimicrobials results in synergistic activity.^{33,34} Synergy occurs when two agents working in concert exert a greater than additive effect, resulting in combinations that are more potent than equivalent doses administered individually, further reducing potential toxicity to the patient and cost of treatment. Herein, NO generated from diazeniumdiolate-modified proline (PROLI/NO) and AgSD were evaluated alone and in combination against 9 pathogenic microbes using acute (2 h) time–kill viability assays. The synergistic in vitro effect of both agents was assessed for four Gram-negative and five Gram-positive strains of bacteria, including two antibiotic-resistant “super bugs”.

Materials and Methods

Materials. L-Proline, sulfadiazine (SD), and AgSD were obtained from Sigma-Aldrich (St. Louis, MO). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were manufactured by BD (Franklin Lakes, NJ) and purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride, potassium chloride, and sodium phosphate monobasic obtained from Fisher, and sodium phosphate dibasic obtained from Sigma-Aldrich were used to prepare phosphate buffered saline (PBS, $I_c = 0.16$ M, pH = 7.4). Distilled water was purified using the Millipore Milli-Q UV Gradient A-10 system (Bedford, MA) to a resistivity of 18.2 M Ω cm and used to prepare the reagents for bactericidal assays. Materials used for growing pathogens and/or evaluating antimicrobial activity were exposed to UV radiation or sanitized in an autoclave prior to use, unless purchased sterile. Argon, NO, nitrogen (N₂), and a NO standard (25.7 ppm in N₂) were purchased from National Welders (Raleigh, NC).

Synthesis and Characterization of PROLI/NO. The synthesis protocol reported by Saavedra et al. was used in the preparation of PROLI/NO.³⁵ Briefly, 10 g of L-proline was dissolved in 39 mL of 25% sodium methoxide in methanol. An additional 20 mL of methanol was added, and the solution was placed into a custom NO reaction bomb, which was then purged with Ar. The proline solution was then exposed to 5 atm of NO for 3 days to form PROLI/NO as a white precipitate in the methanol. After the bomb was purged with Ar, the precipitate was isolated by vacuum filtration, washed with ether, and dried under vacuum. The white solid (PROLI/NO) was divided into small aliquots (<1 g) and stored over desiccant at –20 °C.

Nitric oxide release from PROLI/NO was characterized using a chemiluminescence NO analyzer (Sievers model 280, Boulder, CO). Briefly, a known quantity of PROLI/NO was inserted into a glass flask containing PBS at 37 °C. Nitric oxide generated into solution via diazeniumdiolate NO donor decomposition was carried to the analyzer by N₂ bubbling through the solution at a flow rate of 80 mL min^{–1}. The NO analyzer was calibrated using an atmospheric sample passed through an NO zero filter and a 25.7 ppm NO standard. The NO release from PROLI/NO was measured periodically to ensure no significant decomposition of the NO donor over the duration of use.

Bacterial Culture. The microbial strains used in this study were obtained from American Type Culture Collection (ATCC, Manassas, VA). The ATCC identification number for each strain was as follows: *Escherichia coli* JM109 (53323), *E. coli* O157:H7 (35150), vancomycin-susceptible *Enterococcus faecalis* (VSEF) (29212), vancomycin-resistant *E. faecalis* (VREF) (51299), *Proteus mirabilis* (29906), *Pseudomonas aeruginosa* (19143), methicillin-susceptible *Staphylococcus aureus* (MSSA) (29213), methicillin-resistant *S. aureus* (MRSA) (33591), and *Staphylococcus epidermidis* (35983). Experiments requiring transfer of biohazardous materials were conducted in a dedicated laminar flow hood equipped with a UV lamp. Lyophilized bacteria were reconstituted in TSB and cultured overnight at 37 °C. A 1 mL aliquot of culture was grown in 100 mL of TSB for 2–4 h until reaching an optical density at 600 nm (OD₆₀₀) ~ 0.15–0.3. The resulting culture was stored at –80 °C in 1 mL aliquots. For daily experiments, 1 mL of bacteria culture was grown in 100 mL of TSB overnight at 37 °C. Recultured in fresh TSB the next day, the bacteria were then grown to midexponential phase, as determined by OD₆₀₀ measurements, to approximately 1 × 10⁸ colony forming units (cfu) mL^{–1}. The relationship between the OD₆₀₀ and the concentration of bacteria in the culture suspension was calibrated for each strain using a Spectronic 301 spectrophotometer (Milton Roy, Ivyland, PA) and enumeration of cfu from culture dilutions grown on TSA plates. For single-agent bactericidal assays conducted to determine the bactericidal activity of PROLI/NO, the 1 × 10⁸ cfu mL^{–1} bacterial suspension was diluted 100-fold in TSB to obtain a final concentration of 1 × 10⁶ cfu mL^{–1}. For single-agent bactericidal assays employing AgSD and checkerboard assays, a 50-fold dilution in TSB was performed, resulting in a 2 × 10⁶ cfu mL^{–1} bacterial concentration.

Single-Agent Bactericidal Assays. The bactericidal efficacy of single agents (e.g., AgSD, PROLI/NO) after 120 min of exposure was evaluated against each pathogenic organism using a time–kill protocol. The minimum bactericidal concentration at 120 min (MBC₁₂₀) was defined as the concentration of AgSD or PROLI/NO that resulted in a 3 log reduction in viability for a particular species over 120 min. Each strain of bacteria was tested in triplicate against 5 concentrations each of AgSD and PROLI/NO. To determine the efficacy of Ag⁺, solutions of AgSD in TSB were prepared and added to an equal volume of 2 × 10⁶ cfu mL^{–1}

(35) Saavedra, J. E.; Southan, G. J.; Davies, K. M.; Lundell, A.; Markou, C.; Hanson, S. R.; Adrie, C.; Hurford, W. E.; Zapol, W. M.; Keefer, L. K. Localizing antithrombotic and vasodilatory activity with a novel, ultrafast nitric oxide donor. *J. Med. Chem.* **1996**, *39*, 4361–4365.

bacterial suspension for a final starting inoculum concentration of 1×10^6 cfu mL⁻¹. To evaluate the efficacy of NO, PROLI/NO was preweighed into chilled vials, and the appropriate volume of 1×10^6 cfu mL⁻¹ bacterial suspension was added to obtain the target PROLI/NO concentration.

At each time point (0, 60, and 120 min), a 1:10 dilution of the microbial culture was prepared in PBS and a 100 μ L aliquot of each dilution was spread onto TSA plates and incubated at 37 °C overnight. The number of colonies was enumerated to evaluate cell viability at 0, 60, and 120 min.

To circumvent common pitfalls associated with traditional efficacy techniques, some aspects of the bactericidal assays were modified from standard protocols.³⁶ The most important features involved obtaining bactericidal (rather than inhibitory) concentrations and requiring efficacy over acute treatment windows (2 h). The rationale was to ensure swift and efficient bactericidal efficacy, as these therapeutic parameters discourage the selection of mutated resistant species. Additionally, we observed the susceptibility of a bacterial strain and its dose–response to an agent as a function of time by counting viable colonies at 60 and 120 min, rather than simply observing the all-or-none end point generated at 24 h by inhibitory (turbidity) determinations.

Checkerboard Assay. The checkerboard method³³ was employed to experimentally determine the efficacy of AgSD and PROLI/NO in combination. Modifications analogous to those used in the single-agent bactericidal assays were adopted as described below. Briefly, bacteria (at a final inoculum concentration of 1×10^6 cfu mL⁻¹) were incubated with an array of antimicrobial combinations of AgSD and PROLI/NO for 2 h at 37 °C. The highest concentration for each antimicrobial tested was a 2-fold dilution of the concentration determined in the single-agent assay exhibiting rapid bactericidal activity ($\geq 99\%$). Three additional dosages at stepwise, 2-fold reductions in concentration were evaluated, resulting in 16 total combinations of AgSD and PROLI/NO tested against each strain of bacteria. For organisms that were particularly susceptible to the combination (i.e., MSSA, VSEF, VREF), lower concentrations of each agent were selected to probe the synergistic limit. Viable colonies were enumerated at 0 and 120 min. The fractional bactericidal concentration index at 120 min (FBC₁₂₀) was calculated using eq 1,

$$\text{FBC}_{120} = \frac{\text{MBC}_{120\text{AB}}}{\text{MBC}_{120\text{A}}} + \frac{\text{MBC}_{120\text{BA}}}{\text{MBC}_{120\text{B}}} \quad (1)$$

adapted from the fractional inhibitory concentration index (FIC) reported by Elion et al.,³⁷ where MBC_{120A} and MBC_{120B} are the values determined for agents A and B, respectively, in the single-agent assay; and MBC_{120AB} and

Table 1. Minimum Bactericidal Concentrations of PROLI/NO and AgSD Required for a 3 log Reduction in Viable Bacteria after 120 min of Exposure

species	Gram class	MBC ₁₂₀ (g L ⁻¹)	
		AgSD	PROLI/NO
<i>E. faecalis</i>	+	2.00	72
VREF	+	4.00	60
<i>S. aureus</i>	+	0.600	48
<i>S. epidermidis</i>	+	0.100	36
MRSA	+	3.20	36
<i>E. coli</i> (JM109)	–	0.050	1
<i>E. coli</i> (O157:H7)	–	0.200	24
<i>P. aeruginosa</i>	–	0.020	8
<i>P. mirabilis</i>	–	0.100	12

MBC_{120BA} are the concentrations of agent A and B that constituted the most effective bactericidal combination as determined by a 3 log reduction of viability. Synergy assays were conducted in three independent experiments for each strain of bacteria. A FBC₁₂₀ < 0.5 was defined as synergistic, while a FBC₁₂₀ < 0.25 was considered highly synergistic.

Results

Bactericidal Activity of AgSD and PROLI/NO Independently. The bactericidal activity of AgSD and PROLI/NO were evaluated against four Gram-negative (*E. coli* JM109, *E. coli* O157:H7, *P. aeruginosa* and *P. mirabilis*) and five Gram-positive (VSEF, VREF, MSSA, MRSA, and *S. epidermidis*) pathogenic strains of bacteria, including two antibiotic-resistant varieties. The concentrations required for bactericidal efficacy for AgSD against the panel of organisms spanned greater than 2 orders of magnitude (Table 1). *P. aeruginosa* exhibited levels of susceptibility in the low micromolar range (56 μ M, 0.020 g L⁻¹) comparable to previous reports.^{4,38} The bactericidal concentration for *E. coli* (O157:H7) was a full order of magnitude greater (560 μ M, 0.20 g L⁻¹) than *P. aeruginosa*, while MRSA required a ~5-fold greater dose (8960 μ M, 3.20 g L⁻¹) than the MSSA strain. Vancomycin-resistant *Enterococcus faecalis* required the greatest dose to achieve a 3 log reduction in viability (11200 μ M, 4.0 g L⁻¹).

Bactericidal concentrations of PROLI/NO also varied significantly between pathogens. The Gram-negative species *E. coli* (JM109) and *P. aeruginosa* were killed at the lowest PROLI/NO doses at 1 g L⁻¹ and 8 g L⁻¹, respectively. The most susceptible Gram-positive strains were *S. epidermidis* and MRSA, each requiring 36 g L⁻¹ for a 3 log reduction in bacterial viability (Table 1). VSEF exhibited the greatest tolerance to NO, withstanding concentrations of PROLI/NO up to 72 g L⁻¹.

To verify that dissociated SD or regenerated proline did not contribute to observed cytotoxicity, we examined the

(36) Amsterdam, D. Susceptibility Testing of Antimicrobials in Liquid Media. In *Antibiotics in Laboratory Medicine*, 4th ed.; Lorian, V., Eds.; Williams & Wilkins: Baltimore, 1996; pp 52–111.

(37) Elion, G. B.; Singer, S.; Hitchings, C. H. Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. *J. Biol. Chem.* **1954**, *208*, 477–488.

(38) Rosenkranz, H. S.; Carr, H. S. Silver sulfadiazine: Effect on the growth and metabolism of bacteria. *Antimicrob. Agents Chemother.* **1972**, *2*, 367–372.

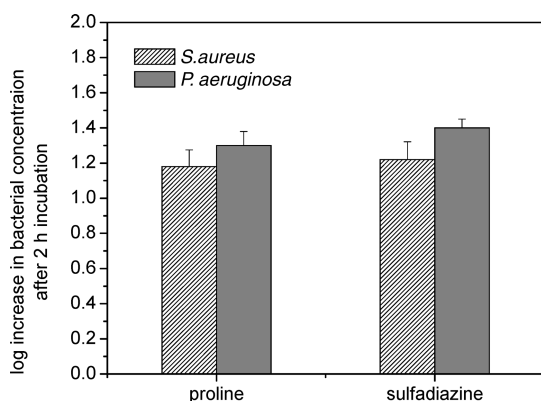


Figure 1. Change in concentration of *S. aureus* and *P. aeruginosa* after 2 h of exposure to proline and sulfadiazine at molar equivalents to the MBC of PROLI/NO and AgSD, respectively, in TSB at 37 °C.

bactericidal activity of these compounds with similar testing protocols. Molar concentrations of SD equivalent to the concentrations required for AgSD cytotoxicity against *S. aureus* and *P. aeruginosa* proved overwhelmingly nontoxic, having neither a static nor cidal effect. In all cases the bacteria continued to multiply during treatment, such that populations increased by more than 1 log over 2 h (Figure 1). Proline exhibited a similar nontoxic effect at concentrations equaling the MBC_{120} of PROLI/NO for the two species. Thus, the bactericidal activity of AgSD and PROLI/NO as single agents can be ascribed to Ag^+ and NO, respectively, under the experimental protocol adopted in this study.

The Gram-positive species studied generally exhibited superior tolerance to the single agents, Ag^+ and NO, when compared to the Gram-negative species. Indeed, the two strains of *E. faecalis* indicated the greatest tolerance to both PROLI/NO and AgSD. The resilience of *E. faecalis* mirrors a report on the efficacy of dilute honey, another broad-spectrum antimicrobial, against a variety of bacterial species, where *E. faecalis* again demonstrated high levels of antimicrobial tolerance.³⁹ Among the Gram-positive species examined in our study, the antibiotic-resistant “super bugs” tended to demonstrate greater tolerance to AgSD, but smaller tolerance to NO than their antibiotic-susceptible congeners. We observed that MRSA exhibited significantly greater tolerance to Ag^+ than its methicillin-susceptible counterpart. Similarly, VREF required twice the dose of AgSD as the vancomycin-susceptible strain. The phenomenon that resistant bacteria selected by exposure to one antimicrobial frequently demonstrate resistance to other agents has been documented repeatedly.^{40–42} For instance, an AgSD-resistant strain of *Enterobacter cloacae* isolated from a burn wound

unit also exhibited a resistance to kanamycin and carbenicillin not expressed by AgSD-susceptible strains.⁴³

Synergistic Activity of AgSD and PROLI/NO in Combination. Measurement of the bactericidal end point over a short treatment duration (2 h) focused the definition of synergy in these experiments to short-term antimicrobial activity, as would be required for most topical applications of these drug combinations in vivo. Using the checkerboard technique, combinations of AgSD and PROLI/NO were screened against *E. coli*, VSEF, VREF, *P. mirabilis*, *P. aeruginosa*, MSSA, MRSA and *S. epidermidis*. Bactericidal synergism ($FBC_{120} < 0.5$) was evident for 4 out of 9 species tested. For one bacterial species, MRSA, the therapeutic combination was highly synergistic ($FBC_{120} < 0.25$). While Gram-positive bacteria were the least susceptible to both AgSD and PROLI/NO as individual antimicrobial agents, the combination of AgSD and PROLI/NO was in fact synergistic against these same species, with the exception of *S. epidermidis*. For example, MRSA and both *E. faecalis* strains demonstrated high tolerance to each agent individually, but suffered the highest degree of susceptibility to the combination of AgSD and PROLI/NO, requiring only 6.25–12.5% of either agent in combination compared to the concentrations required for either of the agents alone. The MSSA strain also exhibited a high degree of susceptibility to this combination, although MRSA was affected to a greater degree. Of interest, *P. aeruginosa* was the most susceptible to AgSD alone, but synergy was not observed from the combination of AgSD and PROLI/NO.

Table 2 indicates the percent of each individual agent needed to elicit the greatest degree of synergy in combination. For the two most synergistic combinations (in Table 2, first two species listed), the dose of PROLI/NO was reduced to 6.25% values when combined with only 12.5% of the dose of AgSD. The much smaller percentage of PROLI/NO required for combined efficacy indicates the importance of PROLI/NO’s role in catalyzing the synergistic mechanism of action. Again using the checkerboard assay, the impact of varying the sequence of addition and spacing between agents was tested (Table 3). PROLI/NO (6 g L⁻¹) and AgSD (0.200 g L⁻¹) were added either simultaneously or at intervals of 15, 30, and 45 min using *S. aureus* as a test organism.

(39) Kwakman, P. H. S.; Van den Akker, J. P. C.; Güçlü, A.; Aslami, H.; Binnekade, J. M.; de Boer, L.; Boszhard, L.; Paulus, F.; Middelhoek, P.; te Velde, A. A.; Vandenbroucke-Grauls, C. M. J. E.; Schultz, M. J. Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. *Clin. Infect. Dis.* **2008**, *46*, 1677–1682.

(40) Akimitsu, N.; Hamamoto, H.; Inoue, R.; Shoji, M.; Akamine, A.; Takemori, K.; Hamasaki, N.; Sekimizu, K. Increase in resistance of methicillin-resistant *Staphylococcus aureus* to β -lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals. *Antimicrob. Agents Chemother.* **1999**, *43*, 3042–3043.

(41) Datta, N.; Faiers, M. C.; Reeves, D. S.; Brumfitt, W.; Orskov, F.; Orskov, I. R factors in *Escherichia coli* in faeces after oral chemotherapy in general practice. *Lancet* **1971**, *1*, 312–315.

(42) Levy, S. B.; Marshall, B.; Schluederberg, S.; Rowse, D.; Davis, J. High frequency of antimicrobial resistance in human fecal flora. *Antimicrob. Agents Chemother.* **1988**, *32*, 1801–1806.

(43) Rosenkranz, H. S.; Coward, J. E.; Wlodkowski, T. J.; Carr, H. S. Properties of silver sulfadiazine-resistant *Enterobacter cloacae*. *Antimicrob. Agents Chemother.* **1973**, *5*, 199–201.

Table 2. Typical Concentrations of AgSD and PROLI/NO That Elicited Optimum Synergistic Effect, the Correlating Fold Decrease over the Respective Values Required for Bactericidal Activity Alone, and the Fractional Bactericidal Concentration (FBC₁₂₀) for Each Species^a

species	AgSD		PROLI/NO		FBC ₁₂₀
	combination (g L ⁻¹)	fraction (%)	combination (g L ⁻¹)	fraction (%)	
MRSA	0.400	12.5	2.25	6.25	0.23
VREF	0.500	12.5	3.75	6.25	0.31
<i>E. faecalis</i>	0.250	12.5	9.0	12.5	0.33
<i>S. aureus</i>	0.100	16.7	12.0	25	0.42
<i>E. coli</i> (O157:H7)	0.050	25	6.0	25	0.53
<i>P. mirabilis</i>	0.050	50	0.75	6.25	0.56
<i>P. aeruginosa</i>	0.005	25	2.0	25	0.63
<i>E. coli</i> (JM109)	0.0125	25	0.25	12.5	0.67
<i>S. epidermidis</i>	0.050	50	18.0	50	1.00

^a The dashed line demarks the transition from synergistic to indifferent.

Table 3. Change in *S. aureus* Viability at 120 min after Exposure to 6 g L⁻¹ PROLI/NO (PNO), 0.200 g L⁻¹ AgSD, Varying the Sequence and Interval between Additions^a

agent 1	addition interval (min)	agent 2	bacterial viability change (log change)
AgSD	45	PNO	-1.61
AgSD	30	PNO	-2.35
AgSD	15	PNO	-2.64
PNO	45	AgSD	-0.51
PNO	30	AgSD	-0.37
PNO	15	AgSD	-2.55
AgSD	0	PNO	-2.46
PNO	n/a	N/A	+0.11
AgSD	n/a	N/A	-1.66

^a Addition interval denotes time period between the addition of the first and second agents.

The potential mechanistic implications of these studies are discussed below.

Discussion

Bactericidal Efficacy of PROLI/NO and AgSD. To understand the synergism observed in this study, it is first necessary to parse out the mechanisms of AgSD and PROLI/NO action. The various roles of NO in killing bacteria have been reviewed previously.^{10,14,17,44} As an indiscriminate and short-lived reactant, the target sites available in the immediate vicinity of NO (i.e., thiols, amines, transition metals, small molecules) play a key role in determining the type of antimicrobial action rendered. The NO donor used in our study (PROLI/NO) releases NO rapidly upon exposure to aqueous solution ($t_{1/2} = 37$ s, Figure 2)^{35,45} where NO and its reactive intermediates likely modify biomolecules on the exterior of bacteria, particularly membrane-bound proteins

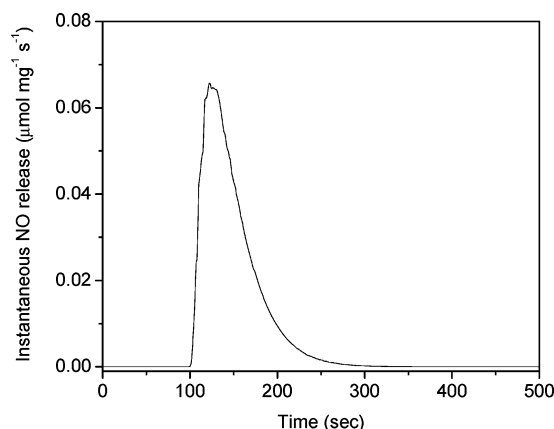


Figure 2. Instantaneous NO release from 0.133 mg of PROLI/NO added to PBS (pH 7.4) at 37 °C.

and lipids. Reaction with O_2^- forms $ONOO^-$, a strong oxidant that can degrade membranes through lipid peroxidation and oxidize nearby proteins, compromising cellular integrity. Additionally, NO is a lipophilic, uncharged, diatomic molecule that readily diffuses across lipid membranes where it may react with intercellular proteins and DNA, disrupting crucial cellular processes. Thus, NO is both an extra- and intracellular threat to bacteria.

Despite its broad application for treating chronic and burn wounds topically, AgSD's mechanism(s) of action remains uncertain. In aqueous media, AgSD gradually dissociates into Ag^+ and SD. Ionic silver reacts directly with thiol-containing amino acids.⁴⁶ Mechanistic studies have shown that treatment with Ag^+ affects DNA replication and cellular respiration, among other functions.^{1–6} A member of the sulfonamide family of semisynthetic antibacterial agents, SD is a biocide in its own right that can interfere with folate synthesis by competitively binding the enzyme dihydropteroate synthase

(44) De Groote, M. A.; Fang, F. C. NO inhibitions: antimicrobial properties of nitric oxide. *Clin. Infect. Dis.* **1995**, 21 (Suppl. 2), S162–S165.

(45) Deupree, S. M.; Schoenfish, M. H. Morphological analysis of the antimicrobial action of nitric oxide on Gram-negative pathogens using atomic force microscopy. *Acta Biomater.* **2009**, 5 (5), 1405–1415.

(46) Liao, S. Y.; Read, D. C.; Pugh, W. J.; Furr, J. R.; Russell, A. D. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. *Lett. Appl. Microbiol.* **1997**, 25, 279–283.

within the cytoplasm of bacteria.⁴⁷ This process is specifically detrimental to bacteria, as higher organisms obtain this metabolite through dietary ingestion. At physiological pH ~ 7 , SD is negatively charged ($pK_a = 6.48$)⁴⁸ and less likely to diffuse across biological membranes to access its intracellular target.⁴⁷ Despite the potential antimicrobial activity of SD, it is generally believed that Ag^+ serves as the primary biocide upon AgSD dissociation, at least for topical applications.⁴⁹ This phenomenon was verified in our experiments by showing that treatment with SD alone at concentrations equivalent to the MBC of AgSD did not affect the viability of any of the organisms studied (Figure 1).

As evidenced by the ineffectiveness of SD, the ability of a biocide to access target sites is imperative for antimicrobial efficacy. The outer membrane characteristic of Gram-negative species acts as a particularly efficient permeability barrier, conferring intrinsic resistance to host defense mechanisms, bile salts and digestive enzymes, and many biocides that are effective against other types of bacteria.⁵⁰ In the absence of differences in specificity, Gram-negative bacteria frequently exhibit improved tolerance to agents that function intracellularly compared to Gram-positive species.⁵¹ Hence, the greater efficacy of Ag^+ and NO (individually) observed against Gram-negative species may indicate that important targets of these biocides reside on the exterior of the bacteria where the rate of passive diffusion is less important. When considering the reactivity of Ag^+ , NO, and NO-derived byproducts, this is not entirely surprising. Empirical evidence drawn from morphological observation supports this hypothesis. For example, electron micrographs of AgSD-treated *P. aeruginosa* and *Enterobacter cloacae* have indicated altered cell wall morphology, while resistant species did not show any changes.^{43,52}

Proposed Mechanisms for the Synergistic Action of PROLI/NO and AgSD. Our control experiments clearly indicate that Ag^+ and NO are the bactericidal agents eliciting synergy. Two possible cooperative mechanisms seem plausible based on the cumulative knowledge of Ag^+ and NO activity, neither of which is mutually exclusive. Agents that act by disrupting the structure of the lipid bilayer or otherwise compromising the cell wall, independent of the level of individual bactericidal activity, should in principle work synergistically when combined with a second agent whose activity is frustrated by low levels of permeability. We have

previously reported that NO is a potent membrane degradation agent, damaging the bacterial membrane of Gram-negative species. *Pseudomonas aeruginosa* treated with NO were imaged with atomic force microscopy, revealing widespread structural deformation and collapse of the bacterial membrane.⁴⁵ These results were further corroborated by the observed penetration of propidium iodide, a fluorescent dye that will only penetrate compromised cell membranes, upon exposure of *P. aeruginosa* to NO.²⁴ Synergistic mechanisms between cell wall- and cytoplasm-active agents have been repeatedly demonstrated (e.g., the improved permeability of streptomycin into *E. faecalis* in concert with cell wall-active agents such as penicillin and vancomycin),^{53,54} and a similar phenomenon has been shown against other Gram-negative and -positive species.³⁴ We hypothesize that such synergism may occur if cell wall or membrane damage elicited by Ag^+ and, particularly, NO significantly increases the permeability of the bacteria cell wall. Both of the antimicrobial agents evaluated in our study have intracellular activity that would be expected to be enhanced by a faster rate of entry into the bacteria. Furthermore, increased permeability of the lipid membrane would improve intracellular access for anionic SD compounds.

Among other bactericidal mechanisms, Ag^+ has been implicated in disruption of cellular respiration. The uncoupling of the respiratory chain initially stimulates respiration as bacteria attempt to regenerate the declining proton gradient across the membrane. Holt and Bard showed that $AgNO_3$ inhibited the respiratory chain by preventing the transport of protons outside of the cell.⁶ One important implication of their study was the probable accumulation of reactive oxygen species such as O_2^- and OH^- at the membrane. The bactericidal efficacy of NO would increase with the number of reactive byproducts produced (i.e., peroxynitrite).

The underlying synergistic mechanism may be indirectly probed by altering the sequence of addition. If decoupling of cellular respiration plays an important role in the synergistic mechanism, then the action of Ag^+ must precede that of the highly reactive NO produced from rapidly decomposing PROLI/NO unless increased cellular permeability is the major contributing factor to the synergistic activity. In the latter case, the PROLI/NO likely elicits the degradation of membrane lipids prior to the subsequent contribution from increased intracellular access of both Ag^+ and NO. A series of bactericidal assays were thus performed varying both the order and time of addition for each bactericidal agent (Table 3). The addition of PROLI/NO prior to AgSD within a 15 min window and AgSD prior to PROLI/NO within a 30 min window resulted in the greatest synergistic effect, suggesting that the order of addition is

(47) Zarfl, C.; Matthies, M.; Klasmeier, J. A mechanistical model for the uptake of sulfonamides by bacteria. *Chemosphere* **2008**, *70*, 753–760.

(48) Sakurai, H.; Ishimitsu, T. Microionization constants of sulpho-namides. *Talanta* **1980**, *27*, 293–298.

(49) Klasen, H. J. Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns* **2000**, *26*, 117–130.

(50) Nikaido, H.; Vaara, M. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **1985**, *49*, 1–32.

(51) Maillard, J.-Y. Bacterial target sites for biocide action. *J. Appl. Microbiol.* **2002**, *92* (s1), 16S–27S.

(52) Coward, J. E.; Carr, H. S.; Rosenkranz, H. S. Silver sulfadiazine: Effect on the ultrastructure of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **1973**, *3*, 621–624.

(53) Moellering, J., R. C.; Weinberg, A. N. Studies on antibiotic synergism against enterococci. II. Effect of various antibiotics on the uptake of ^{14}C -labeled streptomycin by enterococci. *J. Clin. Invest.* **1971**, *50*, 2580–2584.

(54) Moellering, J., R. C.; Wennersten, C.; Weinberg, A. N. Studies on antibiotic synergism against enterococci. I. Bacteriologic studies. *J. Lab. Clin. Med.* **1971**, *77*, 821–828.

not important within a narrow time period. Antimicrobial activity was diminished when AgSD was added >30 min prior to PROLI/NO, perhaps due to reduced potency of the AgSD in solution before any action on the cell walls by PROLI/NO. Similarly, antimicrobial efficacy was reduced when PROLI/NO was added >15 min prior to AgSD, likely due to the short half-life of NO at physiological pH (~40 s). We hypothesize that at additional periods beyond 15 min for PROLI/NO and 30 min for AgSD, either cell division may result in new, nondamaged cells or cell wall repair occurs despite the short-lived presence of sub-bactericidal doses of NO.⁵⁵ Any new or repaired cells would then be unaffected by the addition of sub-bactericidal doses of AgSD, allowing normal proliferation to occur.

To evaluate the potential effect of increased SD permeability, *S. aureus* bacteria were treated with a range of SD concentrations in combination with doses of PROLI/NO at one-half and one-quarter of the respective bactericidal levels. No enhanced activity was observed for *S. aureus*, indicating that SD is not involved in the primary synergistic mechanism. Even the lowest concentration of SD evaluated (0.105 g L⁻¹) was almost 3-fold greater than the equivalent concentration of AgSD required for maximum cooperativity against *P. aeruginosa* (0.005 g L⁻¹) with a 12 g L⁻¹ dose of PROLI/NO. These results indicated that combination treatment with NO and SD was not particularly effective against *S. aureus* over a 2 h time frame, although some enhanced effect may be expected to occur over longer periods as endogenous folate reserves are depleted and cellular activity decreases due to starvation. The primary mechanism of the acute synergy observed at 2 h involves both NO and Ag⁺, and the evidence revealed in this study indicates that increased intracellular concentrations of these two agents following membrane damage may play a significant role in the observed synergistic activity.

(55) Chilton, P.; Isaacs, N. S.; Manas, P.; Mackey, B. M. Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. *Int. J. Food Microbiol.* **2001**, *71* (1), 101–104.

Conclusions

The combination of AgSD and PROLI/NO is synergistic across a wide range of Gram-positive bacteria, including antibiotic-resistant “super bugs”. By varying the interval of addition of AgSD and PROLI/NO, we demonstrated that the duration between dosing of individual agents is important in eliciting maximum synergistic activity. When the concentration of AgSD or PROLI/NO is sub-bactericidal, the delay in the addition of the second agent should not exceed some threshold time limit, presumably as the bacteria are capable of repairing the membrane damage or dividing to form healthy cells, even while under stress.

When AgSD and PROLI/NO were evaluated against bacteria individually, Gram-negative bacteria were the most susceptible to treatment, with as little as 0.020 g L⁻¹ AgSD and 1 g L⁻¹ PROLI/NO required for a 3 log reduction in viability of *P. aeruginosa* and *E. coli* (JM109), respectively. Gram-positive bacteria were the most susceptible to the cooperative effect of the combination of AgSD and PROLI/NO. In the case of MRSA, only 12.5% of the AgSD and 6.25% of the PROLI/NO was required to be bactericidal in combination compared to the concentrations needed when used individually. The evidence presented herein provides an impetus to further investigate the clinical uses of NO in combination with AgSD and other antibiotics. Possessing the ability to rapidly eradicate a wide range of bacterial species, such combinations may be particularly useful in topical wound treatments due to the potential for treating polymicrobial and antibiotic-resistant infections while reducing selection for resistant species. Further mechanistic studies aimed at elucidating the synergistic action of AgSD and NO would provide a strong foundation for developing and improving additional combination therapies. In addition, studies examining both the cytotoxicity and in vivo efficacy of the therapy are necessary prior to clinical application.

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